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DNA amplification at chromosome position 20q13.2 is common in breast cancer, correlates with poor prognosis, and may reflect the presence of an important oncogene that has not been previously characterized. A candidate oncogene in this region, ZNF217, whose level of expression matches degree of amplification, has been identified through gene mapping and expression studies. To begin to understand how ZNF217 overexpression contributes to breast cancer progression, in vitro studies are being performed to determine how retrovirally transduced ZNF217 alters the phenotype of human mammary epithelial cells (HMEC) from normal tissue. Several biological assays useful in distinguishing normal HMEC from immortally and tumorigenically transformed cells are being used to compare the ZNF217-transduced cells with control cells. To assay the effect of ZNF217 expression on replicative lifespan, parallel cultures were infected with ZNF217 or a control virus, and then were passaged up to, or when indicated, beyond the passage at which control cultures senesced or died. Preliminary data indicates that amplification/overexpression of ZNF217 may be selected for during human breast cancer progression because it effectively immortalizes susceptible HMEC. Overcoming replicative senescence may be necessary for such cells to accumulate the multiple errors necessary for invasion and metastasis.

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INTRODUCTION

DNA amplification at chromosome position 20q13 is common in breast cancer, correlates with poor prognosis, and may reflect the presence of an important oncogene which has not been previously characterized. A candidate oncogene in this region, ZNF217 (previously designated ZABC1), whose level of expression matches degree of amplification, has been identified through gene mapping and expression studies. In order to begin to understand how ZNF217 overexpression contributes to breast cancer progression, in vitro studies are being performed to determine how retrovirally transduced ZNF217 alters the phenotype of human mammary epithelial cells (HMEC) from normal tissue. Several biological assays useful in distinguishing normal HMEC from immortally and tumorigenically transformed cells are being used to compare the ZNF217-transduced cells with control cells. Alteration of specific phenotypic properties in HMEC overexpressing ZNF217 will provide direct evidence of the gene's oncogenic potential, and provide information about the biochemical pathways affected.

BODY

The coding sequence of ZNF217 was subcloned into a standard, widely-used retroviral vector, LXSN [1], for efficient uptake and expression in HMEC. High titer amphotropic stocks of ZNF217 and control retrovirus were prepared using a transient packaging system [2] and used to infect recipient HMEC cultures. Northern analysis showed clear evidence of ZNF217 mRNA overexpression (Fig.1), and immunoblot analysis using a crude antibody preparation showed evidence of ZNF217 protein expression, in the infected cells (Fig. 2). A separate experiment employing a ZNF217-EGFP fusion construct in a plasmid vector showed preferential nuclear localization of fluorescent signal in transfected COS7 cells, confirming the presence of a functional nuclear localization signal in the ZNF217 sequence (Fig. 3). Interestingly, the ZNF217-EGFP fusion protein exhibited a particularly punctate, speckled pattern of fluorescence in some nuclei.

Since a current major interest in our lab is defining the mechanisms that enable normal, finite lifespan HMEC to overcome replicative senescence and acquire the ability to proliferate indefinitely, and since evidence existed that 20q amplification is associated with overcoming replicative senescence, we looked to see whether overexpression of ZNF217 had an effect on replicative lifespan. The successful identification of a gene that contributes to extended or indefinite lifespan depends upon the use of a target cell population where there is a reasonable likelihood that a single change can alter replicative lifespan. We knew from our previous experiments that cells with extended life (EL) compared to untreated controls could be obtained from reduction mammoplasty-derived HMEC cultures exposed to a chemical carcinogen [3], and that very rarely, such EL cultures yield cell lines with indefinite lifespan. Alternatively, when grown in serum-free MCDB 170 medium, untreated reduction mammoplasty derived HMEC cultures give rise to a "self-selected" population capable of long-term growth before reaching replicative senescence [4]. Neither the EL nor the post-selection HMEC express p16, a negative modulator of Rb phosphorylation, because of methylation of the promoter or mutations sustained in the gene itself [5]. Both the EL and the post-selection HMEC can be immortalized in the absence of changes in Rb [6,7] or p53 [3,8]. We chose to begin our transgene experiments with carcinogen-treated, 184Aa EL and post-selection finite lifespan strain 184 HMEC to assay ZNF217 effects on replicative lifespan. In these experiments, cells were infected with ZNF217 or a control virus, and then parallel cultures were passaged up to or when indicated, beyond, the passage at which control cultures senesced or died. Since we found that expression of ZNF217 did not lead to immediate heterogeneity in HMEC morphology or growth rate, the G418-selected cells were pooled in these studies.

Four independent experiments have been performed to date using 184Aa EL cultures. 184Aa cultures infected with ZNF217 retrovirus generally grew slower than parallel cultures infected with control virus during the first 3 to 4 passages after infection. The 184Aa-ZNF217 mass cultures continued to grow, however, after the control mass cultures had clearly senesced around passage 14 (Fig.4A & Fig. 5A,B). The 184Aa-ZNF217 cultures were marked at this point by slow, heterogeneous growth. Areas of small, mitotic, senescence associated beta-galactosidase (SA-ßgal; [9]) negative cells appeared, interspersed with areas of large, flattened, clearly post-mitotic SA-ßgal(+) cells (Fig.5B). Within 3-5 passages, the cultures grew better and became more homogeneous (Fig.5C). In 3/4 experiments, continuously growing, apparently immortal, cell lines were obtained. These experiments were complicated by the fact that some of the control cells in 2 of the experiments gave rise to clonal outgrowths that grew well immediately after their initial appearance, with morphologies very different from ZNF217 and other control cells. We knew from past experiments that insertional mutagenesis by retroviruses could induce indefinite growth in 184Aa in rare instances [10]. Southern analysis of retroviral integration sites in one case confirmed that the 184Aa cells with indefinite lifespan that grew in control dishes originated from a few (≤4) cells at most. In contrast, the 184Aa-ZNF217 cultures with extended growth potential did not arise as clonal

outgrowths and only became apparent when control cultures became senescent. Two 184Aa-ZNF217 cultures have now been taken past passage 43, and continue to show good uniform growth, suggesting that they have attained full immortalization.

One experiment has been performed thus far using normal finite lifespan HMEC strain 184. 184 cultures infected with ZNF217 retrovirus at passage 12 also grew slower during the first few passages after infection than parallel cultures infected with control virus. Control cultures senesced at passage 20. In contrast, the ZNF217-infected cultures continued to grow (Fig 4B), again slowly and heterogeneously at first (Fig.5E), but better and more homogeneously 4-6 passages later (Fig.5F). These cultures are now at passage 33. In contrast to the carcinogen treated EL cultures, we have never observed spontaneous immortalization or immortalization due to insertional mutagenesis in untreated normal finite lifespan HMEC. Two additional normal finite lifespan cultures, 161 and 239, from different individuals, have been infected with ZNF217 and control viruses to assess the reproducibility of the ability of ZNF217 to extend lifespan in normal HMEC. Although the data is still very preliminary, ZNF217 appears to extend the proliferative lifespan of specimens 161 and 239, as well (data not shown).

The finding that overexpression of ZNF217 can cause immortalization of normal HMEC is a major advance. It indicates that amplification/overexpression of ZNF217 may be selected for during human breast cancer progression because overcoming replicative senescence may be necessary for such cells to accumulate the multiple errors necessary for invasion and metastasis. Only one other cellular oncogene, c-myc, has been noted to cause immortalization of HMEC, and in the case of c-myc, the immortalization is inefficient ([11]; our own unpublished results). While the viral oncogene, HPV16-E6, has been shown to efficiently immortalize normal post-selection HMEC, it is likely that this viral oncogene, unlike cellular genes, has been evolutionarily selected to simultaneously compromise several immortalization suppressing functions [12]. Furthermore, viral oncogenes have not been shown to play roles in most human malignancies, including those of breast.

In post-selection HMEC, fibroblasts, and other cell types, replicative senescence has been correlated with shortened telomeres, while overcoming replicative senescence has been correlated with the acquisition of telomerase, an enzymatic activity that can stabilize or lengthen telomeres. The multi-protein ribonucleoprotein telomerase complex can add telomeric sequences de novo, obviating the end-replication problem in linear chromosomes. Cells maintaining telomerase activity display regulation of telomere length, and can retain indefinite proliferative potential. Telomerase activity is detected in the large majority of human cancer tissues and tumor-derived immortal cell lines, which usually maintain short stable telomeres (mean TRF ~3-7 kb) [13]. In contrast, normal finite lifespan human somatic cells do not display telomerase activity, although low levels are seen in some stem cell populations, and transient high levels can be observed in activated T cells [14]. Although telomerase(-) normal human cells express the RNA component (hTR) of the telomerase complex [15,16], the catalytic component (hTERT) is not expressed [15,16]. Transfection of hTERT into some telomerase(-) human cells [17-19], including post-selection HMEC [7], indicates that it is the limiting component of telomerase activity, and sufficient for producing an indefinite lifespan.

Our previous work examining the steps involved in immortal transformation of benzo(a)pyrene-exposed cultured HMEC showed that acquisition of strong telomerase activity and stabilized telomere lengths did not occur simultaneously with the step that transformed the finite lifespan HMEC to a population with indefinite proliferative potential [20]. Because few individual cells in initial passages of the clonally derived immortalized cultures were capable of indefinite growth, but the mass culture was routinely capable of indefinite growth, we have used the term "conditionally" immortal to describe cells at this stage. Conditionally immortal HMEC, like finite lifespan HMEC, are sensitive to TGFβ-induced growth inhibition; all normal HMEC ultimately growth arrest in its presence. In contrast, fully immortal HMEC and breast tumor-derived lines can maintain proliferation indefinitely in the presence of TGFβ, although often at a slower rate. In our HMEC immortally transformed after carcinogen exposure, acquisition of telomerase activity was closely correlated with subsequent gradual acquisition of TGFβ resistance. We have since shown that transduction of normal and conditionally immortal HMEC with hTERT leads to immediate acquisition of telomerase activity and subsequent acquisition of TGFβ resistance, indicating that the two traits are somehow linked (unpublished data).

The conditionally immortal population still needed to undergo an additional step, which we have termed "conversion," in order to attain uniform unlimited growth. The conversion of telomerase(-), TGFß growth-sensitive, conditionally immortal HMEC to telomerase(+), TGFß growth-resistant, fully immortal HMEC starts to occur in p53(+) cells when telomere lengths fall below a critical value, and is accompanied by slow heterogeneous

growth and accumulation of the CDK inhibitor, p57Kip2 [21]. The conversion process is characterized by a prolonged period of poor, heterogeneous growth, coincidant with high p57 expression and mean terminal restriction fragments (TRF; an indicator of telomere length) < 3 kb, followed by a gradual attainment of uniform good growth ± TGFβ, strong telomerase activity, and absence of p57 expression. In the three p53(+) immortal HMEC lines we have managed to generate by chemical carcinogen treatment and/or insertional mutagenesis, conversion extended over 12-18 months, with uniformly good growing populations present only 20-35 passages after the lines first appeared. The high frequency and incremental nature of conversion in subcloned populations is more consistent with an epigenetic rather than a genetic origin.

Although loss of p53 itself is not necessary for HMEC immortalization, our new data [10] suggests that the total loss of p53 function as part of the immortalization process may produce indefinite lifespan cell lines with significant initial and long-term differences relative to immortal HMEC which retain p53 function. Specifically, we have shown that p53(-/-) HMEC undergo an accelerated conversion to full immortality; telomerase expression is present at early passage levels, mean TRF length does not decline below 3.5 kb, expression of p57 is not detected, and uniform good growth is attained within 10 passages following isolation of these cell lines. Additionally, the p53(-/-) lines demonstrate many initial structural and numerical abnomalities in their karyotypes, as well as genomic instability with continued passage. While total loss of p53 is not necessary for in vitro or in vivo HMEC transformation, it is possible that an activity controlled by the p53 pathway may be compromised in p53(+) immortal HMEC. A more discrete lesion, leaving functional p53 present, would result in a less deranged phenotype than total p53 loss, and could contribute to the generally less aggressive behavior of breast cancers containing wild-type p53.

ZNF217-overexpressing HMEC resemble conditionally immortal HMEC in that they also undergo a heterogeneous growth stage at the time when control cultures are senescing, before acquiring telomerase activity and uniform indefinite growth potential. Preliminary analysis of ZNF217-immortalized 184 and 184Aa cultures indicates that telomerase activity increases incrementally with passage after the point of replicative senescence in control cells is passed (Fig.6). Thus ZNF217 transduction may provide a suitable reproducible system for studying the molecular and biochemical changes that accompany immortalization and conversion of finite lifespan HMEC to a fully immortal phenotype in order to determine whether these changes are amenable to therapeutic intervention.

In the future, we will continue to characterize any additional effects of ZNF217 on phenotypes associated with tumorigenesis. In addition to the stated studies to be performed, we are examining genomic stability, anchorage dependence, and tumorigenicity of the ZNF217 immortalized HMEC. Comparitive genomic hybridization (CGH) is being performed after every 5-10 passages to determine whether ZNF217-induced immortalization is associated with any additional genetic lesions or increased genomic instability. Thus far, analysis of one ZNF217-immortalized 184Aa culture shows that there are some chromosomal changes which accompany the immortalization (Fig.7). This suggests that high ZNF217 expression might promote genomic instability in addition to immortalization in susceptible cells.

KEY RESEARCH ACCOMPLISHMENT

• Overexpression of a retrovirally transduced ZNF217 gene in normal finite lifespan and carcinogen treated extended-life HMEC cultures leads reproducibly to immortalization.

REPORTABLE OUTCOMES

None to date.

CONCLUSIONS

Preliminary data indicates that amplification/overexpression of ZNF217 may be selected for during human breast cancer progression because it effectively extends the replicative lifespan of susceptible cells. Overcoming replicative senescence may be necessary for such cells to accumulate the multiple errors necessary for invasion and metastasis. The data also suggests that ZNF217 overexpressing HMEC may undergo a slow, heterogeneous growth stage similar to that exhibited by HMEC made conditionally immortal by chemical carcinogen treatment. Thus ZNF217 transduction may provide a suitable reproducible system for studying the molecular and biochemical changes that accompany immortalization and conversion of finite lifespan HMEC to a fully immortal phenotype in order to determine whether these changes are amenable to therapeutic intervention.

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APPENDICES

Fig.1. ZNF217 mRNA expression in retrovirally transduced 184 HMEC.

Fig.2. ZNF217 protein expression in retrovirally transduced 184 HMEC.

Fig.3. Nuclear localization of ZNF217-EGFP fusion protein in transfected cells.

Fig.4. Cumulative population doublings by control and ZNF217-transduced HMEC.

Fig.5. Gradual loss of senescence-associated beta-galactosidase expression in ZNF217-transduced HMEC.

Fig.6. Gradual acquisition of telomerase activity in ZNF217-transduced HMEC.

Fig.7. Comparative Genomic Hybridization data for 184Aa-ZNF217 23p.

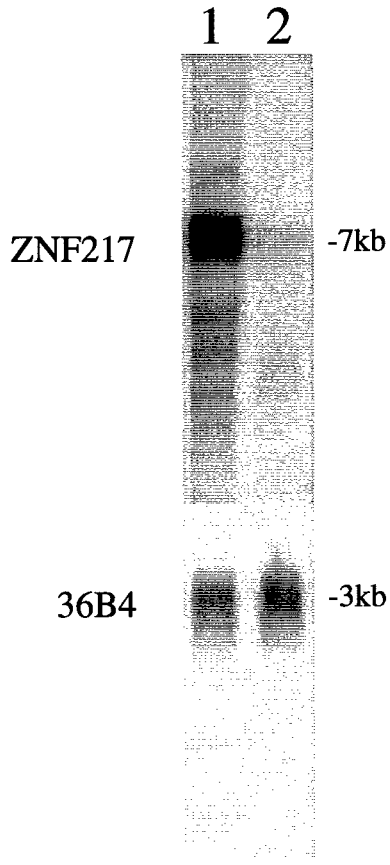

Fig. 1 ZNF217 mRNA expression in retrovirally transduced 184 HMEC. A Northern blot of total RNA from184Aa cells infected with a ZNF217-containing retrovirus (lane 1) or infected with the retroviral vector alone (lane 2) was hybridized with a ZNF217 gene fragment. The bottom panel shows the same Northern blot hybridized to a constitutively expressed endogenous gene encoding a ribosomal acidic protein (36B4).

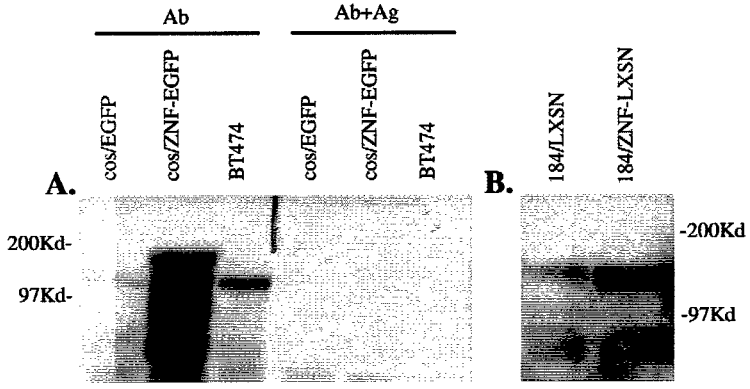

Fig. 2 ZNF217 protein expression in retrovirally transduced 184 HMEC.
A) The specificity of the anti-ZNF217 antibody was demonstrated by duplicate Western blots of whole cell lysates of cos7 cells transfected with a green fluorescent protein (EGFP) construct alone, cos7 cells transfected with a green fluorescent protein-ZNF217 fusion protein (ZNF-EGFP) construct, and endogenous ZNF217-overexpressing human breast tumor cell line BT474. The blot on the left was incubated an anti-ZNF-217 polyclonal antibody alone (Ab), while the blot on the right was incubated with a combination of the polyclonal antibody and the antigen to which the antibody was produced (Ab+Ag).
B) A Western blot of whole cell lysates of 184 HMEC infected with either the retroviral vector LXSN alone or with the retrovirus containing a ZNF217 gene. The predicted size of unmodified ZNF217 is approximately 125Kd.

EGFP ZNF-EGFP

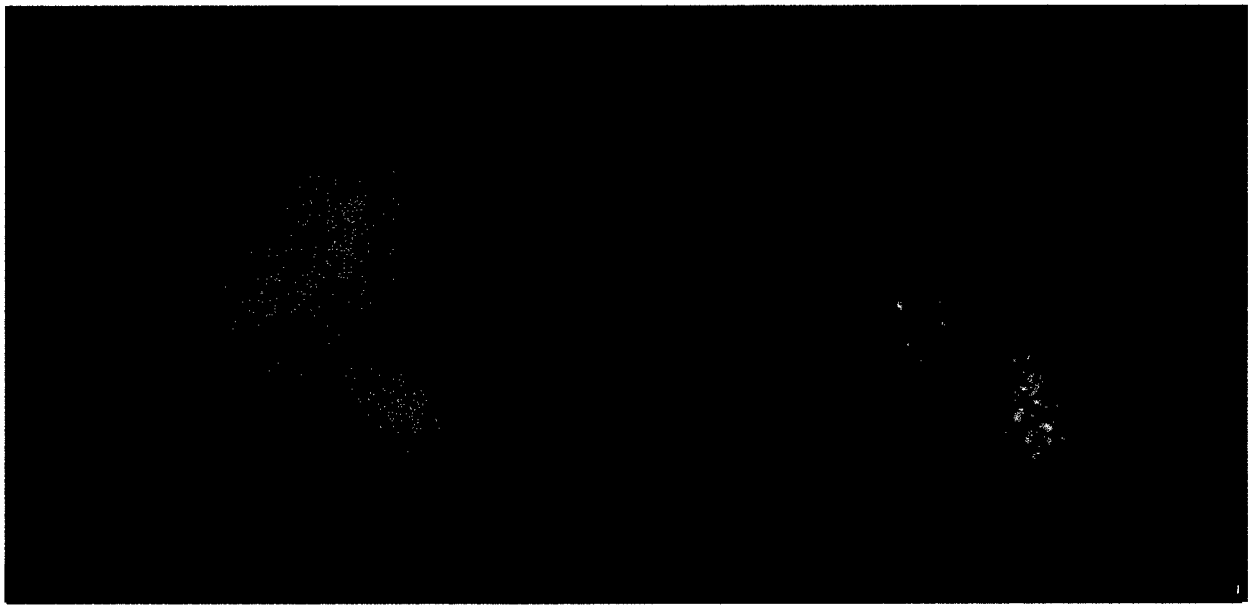

Fig. 3. Nuclear localization of ZNF217-EGFP fusion protein in transfected cells.

Cos7 cells were transfected with plasmids containing genes encoding either green fluorescent protein (EGFP) alone or a ZNF217-green fluorescent protein (ZNF-EGFP) fusion protein. Note that fluorescent signal is detected throughout the cell body of a control cell transfected with EGFP alone (left panel), but that fluorescent signal is confined to the nuclei of two cells transfected with the ZNF-EGFP construct (right panel). The fluorescence is particularly bright in speckles found within the nuclei of the ZNF-EGFP transfectants.

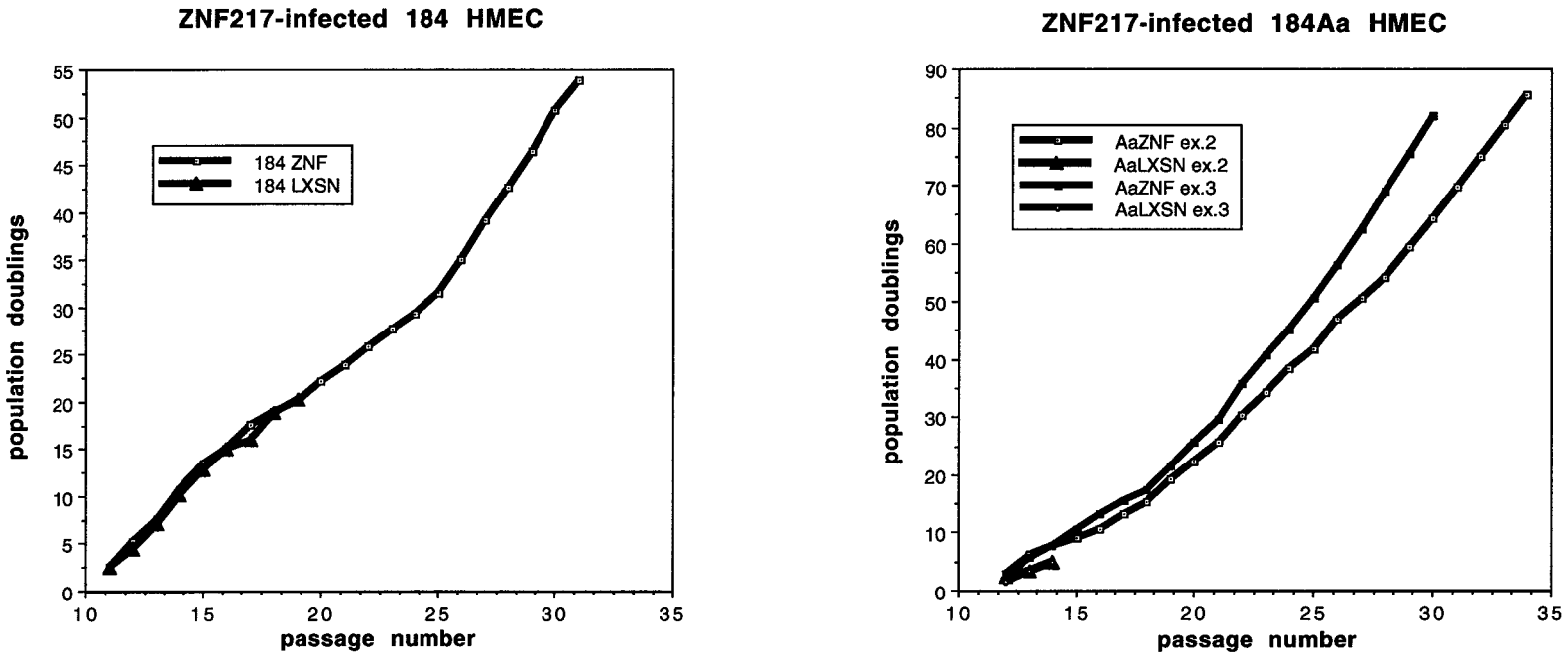

Fig.4 Cumulative population doublings by control and ZNF217-transduced HMEC.
In the left panel the population doublings of 184 cells infected with either vector alone (LXSN) or ZNF217 plotted against the passage number. The LXSN control senesced at passage number 20.
In the right panel, the population doubling of 184Aa cells infected with either vector alone (LXSN) or ZNF217 plotted against the passage number for two independent experiments (ex.2 and ex.3). In ex.2 and ex.3, the LXSN controls senesced at passage number 14. In ex.3, an immortal clone grew out of an otherwise senescent population; the growth of this clone is not plotted here.

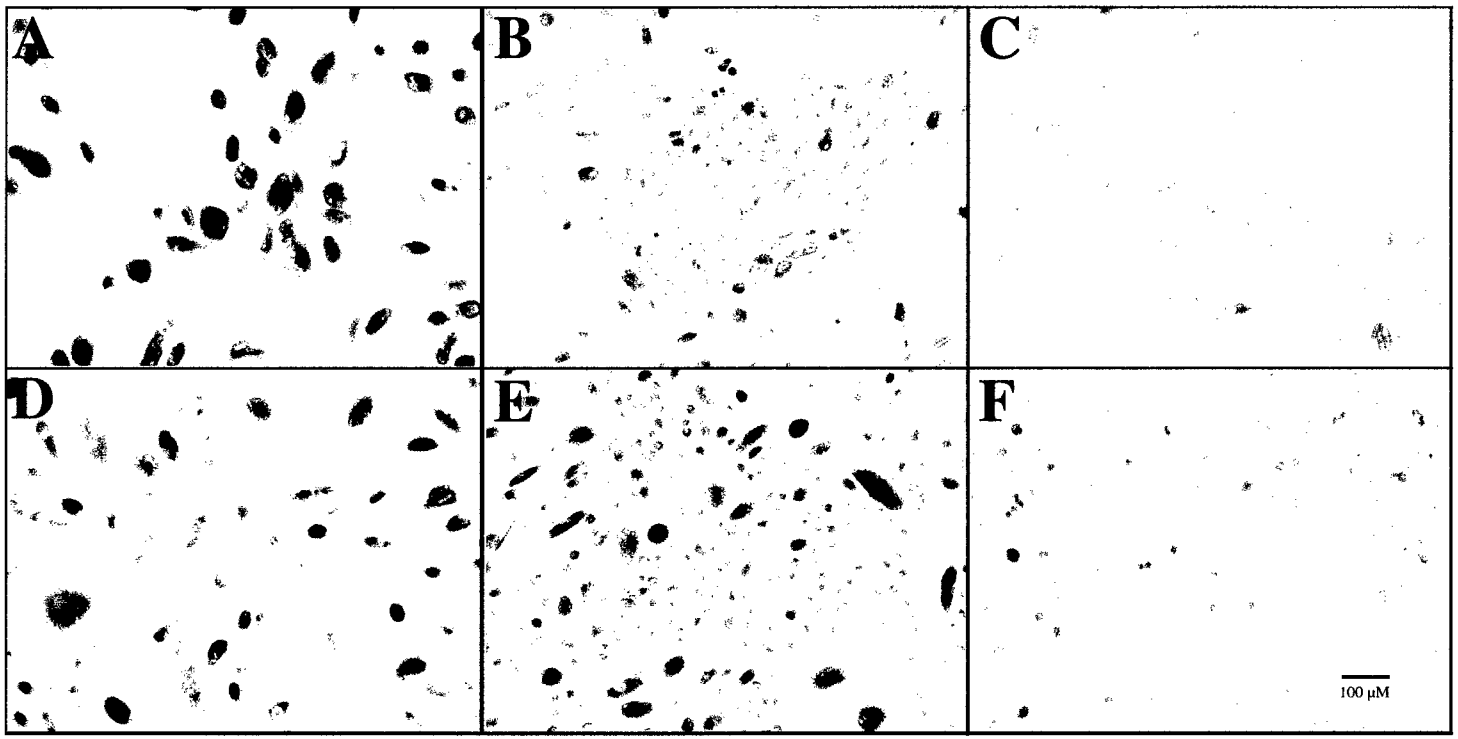

Fig 5. Gradual loss of senescence–associated β-galactosidase expression in ZNF217-transduced HMEC. 184Aa (A-C) and 184 (D-F) HMEC infected with LXSN control (A, D) or ZNF217-containing (B, C, E, F) retrovirus were stained for senescence-associated β-galactosidase activity (pH 6.0) at the following passages: (A) p14, (B) p17, (C) p35, (D) p20, (E) p20, (F) p25. Note the presence of small, senescence–associated β-galactosidase negative cells in HMEC infected with ZNF217 virus.

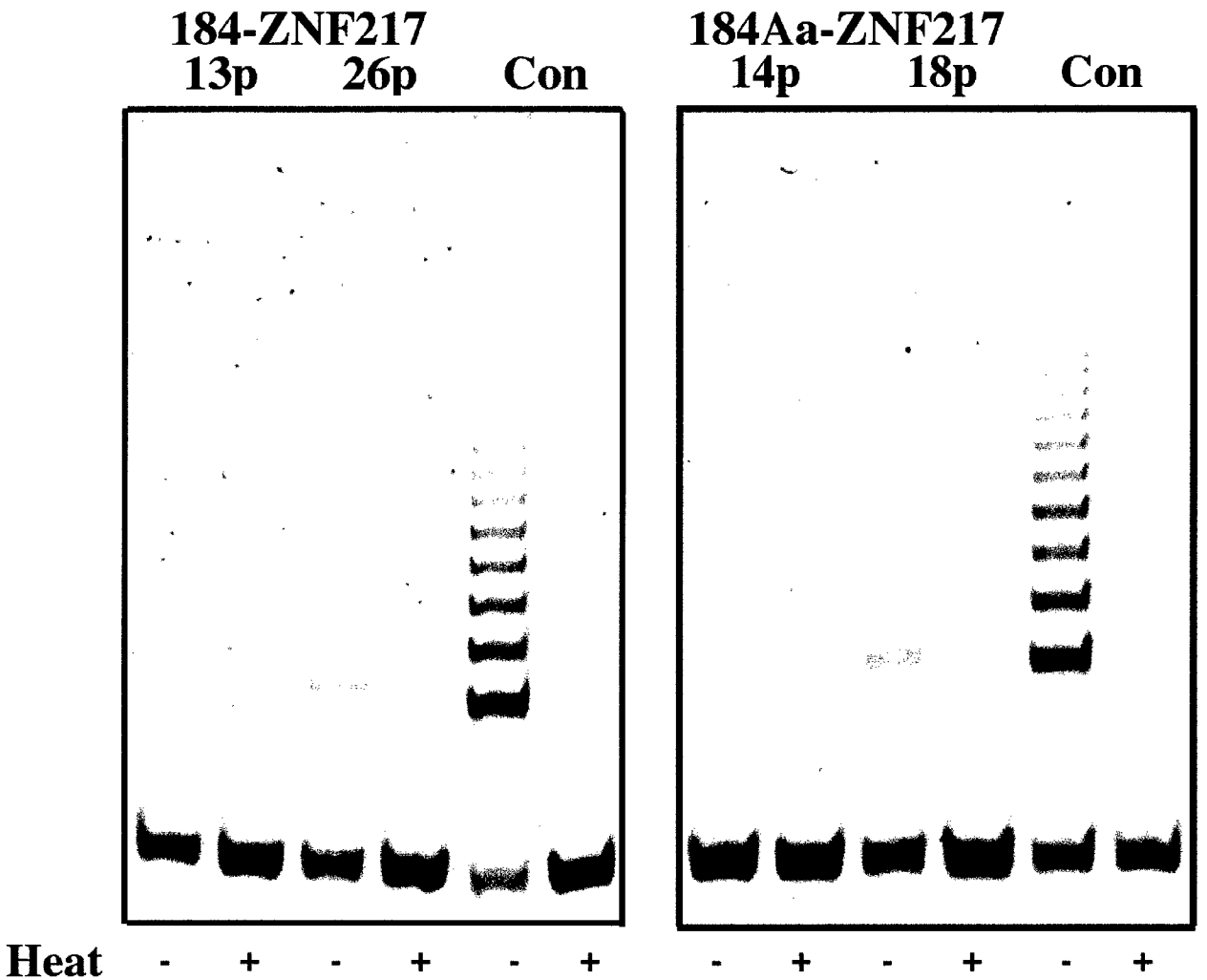

Figure 6. Acquisition of telomerase activity in 184 and 184Aa HMEC infected with ZNF217 retrovirus.

Telomerase activity was measured in 2μg extracts of retrovirally transduced cells at the indicated passages and in a positive control immortalized human kidney cell line (Con) using the TRAP-EZE telomerase detection kit. Heat treated extracts were used as negative controls.

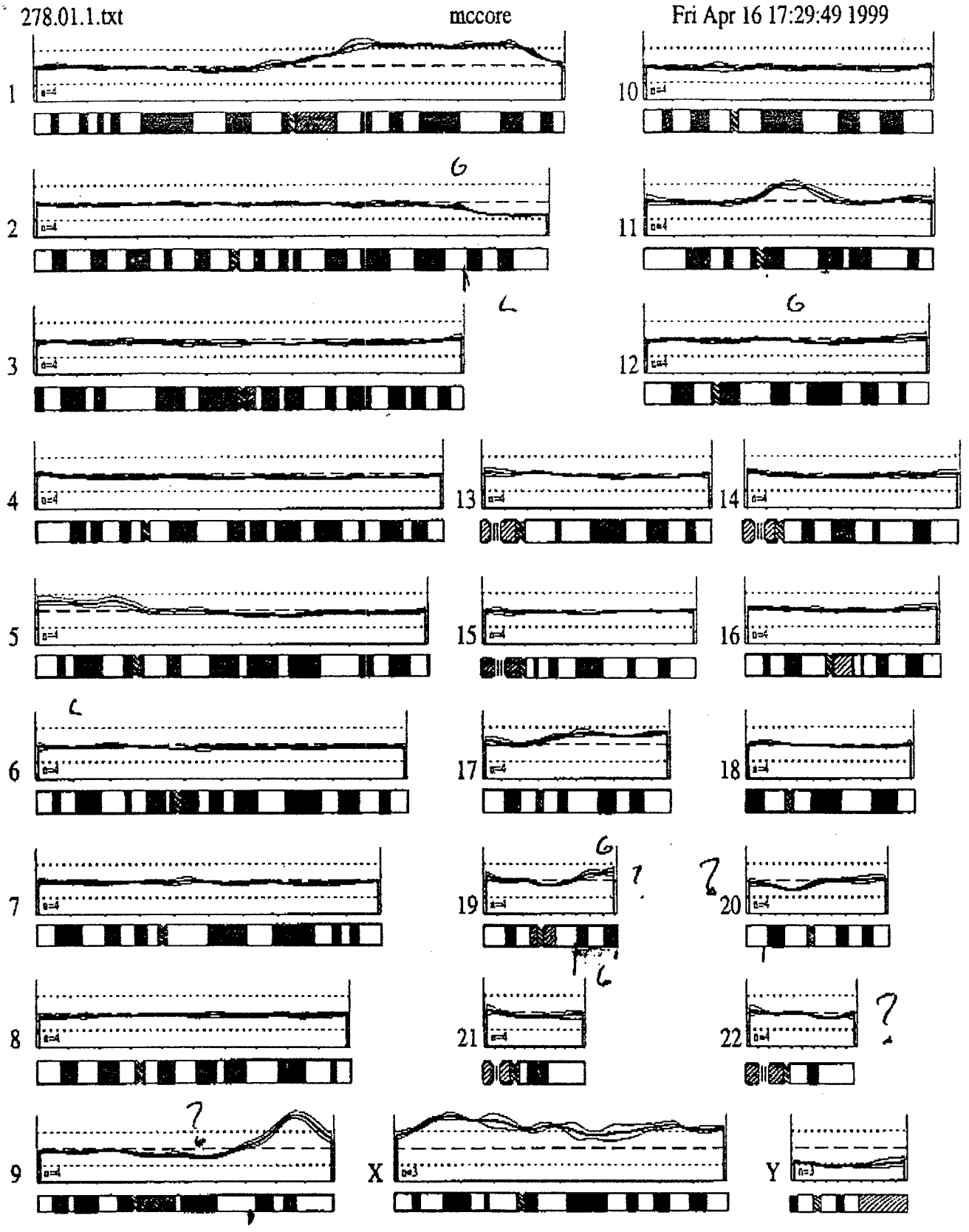

Fig.7.Comparative genomic hybridization reveals DNA-sequence copy-number variation in 184Aa-ZNF217.

Quantitation of green-to-red fluorescence intensities along normal human chromosomes after comparative genomic hybridization with 184Aa-ZNF217 p23 DNA in green and normal DNA in red. The ratios are normalized, so that the average green-to-red ratio for each metaphase cell is 1.00. Note the presence of regional copy-number increases at chromosomes 1q, 5p, 9q, 11q, 17q, 19q(?) and decreases at chromosomes 2q, 9q(?), 20p(?), 22q(?). Question marks denote preliminary data that remain to be verified on additional metaphase spreads.

DEPARTMENT OF THE ARMY
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REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 28 Aug 02

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for this Command. Request the limited distribution statement for the enclosed accession numbers be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

PHYLIS M. RINEHART
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